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Finnish-Type Aspartylglucosaminuria Detected by Oligonucleotide Ligation Assay

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Aspartylglycosaminuria (AGU) is a recessively inherited lysosomal storage disease that occurs with much higher frequency in Finland than elsewhere. AGU is caused by a deficiency in glycosylasparaginase (GA), which results in the accumulation of glycoasparagines in lysosomes. In the Finnish population, a single nucleotide change in the gene encoding GA is responsible for the disease. We have used the oligonucleotide ligation assay (OLA) to detect the mutation in polymerase chain reaction (PCR)-amplified DNA samples from normal, carrier, and affected individuals. Screening for AGU among 415 random Finnish DNA samples with PCR/OLA revealed five carriers of the mutant allele and demonstrated the potential of the method for use in carrier screening. PCR/OLA provides a rapid, reliable, nonisotopic method to detect the mutation responsible for AGU that can readily be applied to large population screening.

Indexing Terms: *lysosomal storage disease/population screening/gene mutations*

Aspartylglycosaminuria (AGU) is an autosomal recessive disorder characterized by serious psychomotor retardation.[4] It arises from the accumulation of aspartylglucosamine as a result of incomplete catabolism of asparagine-linked glycoproteins in lysosomes (*1, 2*). The incidence of this disease outside Finland is very low but in northern and eastern Finland it is estimated to affect between 1 in 4000 and 1 in 7000 individuals, with a carrier rate of 1 in 30 to 1 in 80 (*3, 4*). Individuals with AGU appear to develop normally in the first few years but undergo progressive physical and neurological deterioration after age 5. AGU is currently diagnosed either by assaying for aspartylglucosamine excreted in urine (*5*) or by detecting a deficiency in glycosylasparaginase (GA) activity in cell extracts (*6*) or serum (*7*). However, neither of these tests is suitable for carrier detection of AGU.

A number of molecular defects underlying AGU have been described (for references see *2*). Two single-base changes in the gene coding for GA have been found to be common to all Finnish AGU patients, and each results in the generation of a new endonuclease restriction site as well as an amino acid substitution in the enzyme (*8, 9*). The G → C transversion, which leads to Cys(163) → Ser substitution, results in a deficiency of GA activity (*9*). This mutation generates an *Eco*RI restriction site that can be detected by Southern transfer of genomic DNA (*9, 10*), or in polymerase chain reaction (PCR)-amplified DNA samples by restriction endonuclease digestion (*8, 9*), allele-specific oligonucleotide hybridization (*8*), or minisequencing (*11*). Although rapid, these techniques are not convenient for general population screening because radioisotopes or electrophoresis is required, steps that are not easily amenable to automation and high throughput.

Here we report the detection of both single-base changes associated with Finnish-type AGU with an automated, nonisotopic strategy that combines amplification of target genomic DNA segments by PCR with the discrimination of sequence variants in the amplified DNA products by a colorimetric oligonucleotide ligation assay (OLA) (*12, 13*). OLA takes advantage of the ability of DNA ligases to covalently join adjacent nucleotides only when they perfectly complement a single-stranded DNA template. The OLA includes three oligonucleotides: two 5′-biotinylated allele-specific probes with identical sequences except for the 3′ nucleotide, which varies to complement each of the possible template sequence variants, and one 3′-digoxigenin-labeled reporter probe, which lies directly adjacent to the allele-specific diagnostic probes. In the assay one of the allele-specific probes and a reporter probe are mixed and allowed to hybridize to an amplified DNA template. If there is perfect complementarity between probes and target, the probes are covalently joined by DNA ligase. A single nucleotide mismatch between these probes precludes ligation. Fig. 1 shows an OLA scheme in which normal template DNA is probed for the polymorphism responsible for AGU. In reaction 1, the biotinylated probe that complements normal template is mixed with the reporter probe and template DNA; in reaction 2, the biotinylated probe that complements the mutant DNA is mixed with the reporter probe and template DNA. After ligation the 5′-biotinylated probe is captured on immobilized streptavidin, and unligated reporter probes are washed away. The reactions are then analyzed for the presence of a covalently linked reporter probe by using an ELISA for digoxigenin. In this example, reaction 1 would give a colored signal, indicating a positive ligation event, whereas reaction 2 would give no color, indicating that no ligation occurred between probes.

Materials and Methods

Synthesis of oligonucleotides. Oligonucleotides used for PCR amplification and for the OLA were synthesized on an Applied Biosystems (Foster City, CA) 380A DNA synthesizer by using standard phosphoramidite chemis-

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[4] Nonstandard abbreviations: AGU, aspartylglycosaminuria; GA, glycosylasparaginase; OLA, oligonucleotide ligation assay; and PCR, polymerase chain reaction.
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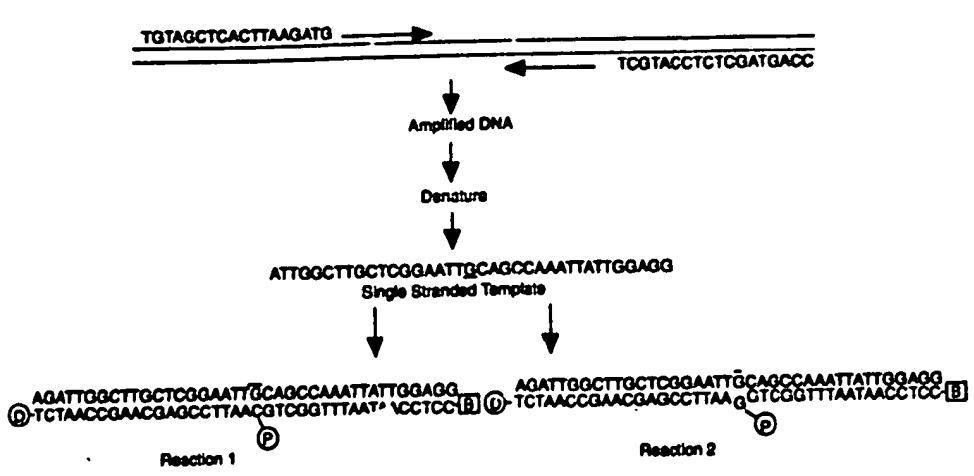

Fig. 1. OLA scheme for detecting the G → C transversion responsible for AGU: normal DNA is probed for the presence of the normal sequence (*reaction 1*) and the mutation (*reaction 2*).

try. Allele-specific probes were modified with a 5′-biotin group as previously described (*14*) and purified by reversed-phase HPLC. After drying, the oligonucleotides were reconstituted with water to a concentration of 5 μmol/L. Reporter probes were chemically modified with 5′ phosphate-ON (Clontech, Palo Alto, CA) and then labeled with dUTP-digoxigenin (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturers' directions. The reaction is complete after 1–12 h at 37°C. The enzyme is inactivated by heating the reaction mixture to 65°C for 15 min, and 80 μL of water is added to give a final concentration of 5 μmol/L.

DNA template amplification. Genomic DNA was isolated from peripheral blood by the sodium dodecyl sulfate–proteinase K/phenol–chloroform extraction method. The DNA template was amplified with primers 5′ TGTAGCTCACTTAAGATG 3′ and 5′ CCAGTAGCTCTCCATGCT 3′, as previously described (*9*). Reactions involved 50 ng of genomic DNA, 100 pmol of each amplification primer, 400 μmol/L of each of the four deoxynucleotide triphosphates, 0.1 U of Taq polymerase, and 1× PCR buffer [10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$] in a total volume of 20 μL. Thermal cycling was performed in microtiter plates on a thermal cycler (Ericomp, San Diego, CA) programmed for an initial 4-min denaturation step at 93°C followed by a temperature step cycle of 93°C (30 s), 55°C (45 s), and 72°C (90 s). A total of 40 cycles were performed, with a final extension step at 72°C for 5 min.

Ligation assays. Ligation reactions were assembled for each allele. The composition of the reaction mixture was: 10× ligase buffer (200 mmol/L Tris, 100 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol), 200 mL/L; 10 mmol/L NAD^+, 200 mL/L; 1 mol/L KCl, 25 mL/L; 1 mL/L Triton X-100 in H_2O, 575 mL/L. For each 300 μL of this reaction, 5 pmol of the appropriate diagnostic oligonucleotide, 5 pmol of the reporter oligonucleotide, and 0.3 μL of thermostable ligase (5000 kU/L; Epicentre, Madison, WI) were added. Amplified template was diluted 1:10 with 10 mL/L Triton in water, and 10 μL of diluted template was mixed with 10 μL of ligation mixtures for each allele. The reactions were placed in the thermocycler and heated: 93°C for 30 s, 58°C for 2 min (10 times). After cycling, reactions were stopped with 10 μL of Triton X-100/EDTA solution (0.1 mol/L EDTA in 1 mL/L Triton X-100 in H_2O). The reaction mixture in its entirety was transferred to a streptavidin-coated, bovine serum albumin-blocked microtiter plate and the product was allowed to accumulate for ≥30 min. The presence of a covalently linked reporter probe was detected by using an ELISA for the digoxigenin reporter.

Results

As a model for the detection of the sequence variations associated with the Finnish-type AGU, OLA was performed on 24 samples of known genotype (*8*) (8 normal, 8 carrier, 8 disease) and on 415 Finnish DNA samples of unknown genotype. OLA results from the known samples are in Fig. 2, which shows three of the possible outcomes for DNA typing by OLA: Normal individuals 1–8 are homozygous for allele 1 and therefore give a positive signal for the normal allele 1 (absorbance reading at 490 nm of 1.398–2.112) and a negative signal for the mutant allele 2 (absorbance reading of −0.033–0.020); individuals 9–16 are heterozygous and show a positive signal for both alleles; and the affected individuals 17–24 are homozygous for the mutant allele 2 and give a positive signal for allele 2 and a negative signal for the normal allele 1. A fourth possibility, negative results for both alleles, usually indicates a failure during PCR amplification. The simple positive/negative format of this readout and the high signal/noise ratios (10:1–200:1) allow rapid genotyping either visually or by automatically calculating the ratio of the absorbance for each allele in the ligation assay with a simple computer program (*13*). Genotyping of 415 random Finnish DNA samples by OLA identified five carriers of the G → C transversion. These same samples also displayed the mutant allele for the neutral G → A transition, a mutation that results in an Arg(161) → Gln substitution and a novel restriction site for *Dde*I. Neither of these mutant alleles was found in any of the normal samples. Restriction enzyme digestion of PCR-amplified DNA (*9*) was used to verify the results of OLA. *Eco*RI digestion of DNA from the five heterozygous individuals produced the full-length 430-bp product as well as a 235-bp frag-

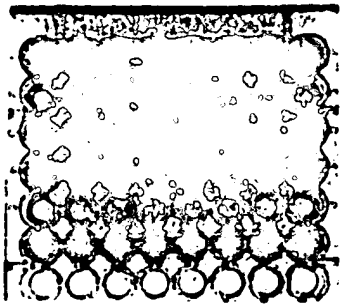

Fig. 2. Amplified DNA targets from 24 blood samples analyzed by OLA for the presence of the G → C transversion, which causes the Cys(163) → Ser change responsible for Finnish-type AGU.
Allele 1 represents the normal allele, *allele 2* the mutant. Samples *1–8* are from normal individuals, samples *9–16* from carriers of the Finnish-type AGU, and samples *17–24* from persons affected by the disease.

ment and a 195-bp fragment resulting from cleavage at the *Eco*RI site created by the G → C mutation. *Dde*I digestion resulted in four bands: 282-bp and 148-bp fragments due to a restriction site in the 3′ intron and two additional fragments at 191 bp and 91 bp due to the restriction site created by the G → A mutation.

Discussion

Ligation reactions provide highly specific and sensitive assays well suited for the detection of the sequence variations that lead to the Finnish-type AGU. When used to assay 415 random Finnish DNA samples, OLA detected five carriers of AGU. This indicates a carrier rate of 1:83, which falls within the range predicted by studies of the frequency of the disease in the Finnish population (*3*, *4*). OLA has several advantages over other diagnostic methods for widespread carrier screening. Coupled with PCR, the assay requires only a small quantity of DNA, obtainable from noninvasive sources such as buccal scrapings or urine. The reagents used in the OLA are stable and nonisotopic and are easily synthesized, yielding $>10^6$ assays from one synthesis of the oligonucleotide reagents. Since the OLA involves a single set of assay conditions for the detection of any polymorphism, screening for AGU alleles can easily be combined with population screening for any number of other genetic diseases. Unlike many methods for analyzing PCR-amplified targets, OLA does not include an electrophoretic step to determine DNA fragment size but rather probes for internal DNA sequences and is therefore unaffected by the formation of spurious amplification products. Most significantly, since the entire assay is performed in microtiter wells, and since the results of the assay can be directly interpreted by a computer, screening by OLA is easily automated. With automation, there is no need for highly skilled technical labor, and >1200 ligation assays per day can be processed by a single technician and a robotic workstation.

Automated analysis of the AGU mutations by PCR/OLA could clearly facilitate screening for at-risk members of families or general carrier screening in Finnish populations. This system is rapid, sensitive, and has a high throughput, which may increase with the development of higher-density formats for immobilizing DNA. Furthermore, with advances in the use of multiple nonisotopic reporter groups (*15*), it may be possible to multiplex the detection of the Finnish-type AGU alleles with detection of mutations causing other diseases common in the population so that several sites can be analyzed simultaneously in a single microtiter well.

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